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(54) Measurement of alpha-amylase activity.

(57) Measurement of α -amylase activity is conducted by using a modified amylose having one carboxymethyl group or a salt thereof per 6 to 36 glucose units in amylose as substrate and using a coupling enzyme of glucoamylase or α -glucosidase to produce glucose and quantitatively determining the glucose produced by a known method. When a special reaction stopper for the coupling enzyme glucoamylase is used, fixed-time assay becomes possible.

MEASUREMENT OF ALPHA-AMYLASE ACTIVITY

1 This invention relates to a process for measuring α -amylase activity using a special substrate and, if necessary, a special stopper, and a reagent composition used for such a process.

5 Measurement of α -amylase activity in a sample, particularly in saliva, pancreatic juice, blood and urine in human living body is important for diagnosis in medical science. For example, α -amylase activity in blood and urine shows a remarkable increase in the
10 case of pancreatitis, cancer of the pancreas, and parotitis compared with normal values.

 Various processes for measuring α -amylase activity have been proposed and can be divided into three groups, that is, an amyloclastic method, a
15 chromogenic method, and a saccharogenic method.

 As to the amyloclastic method, the Caraway method has been most widely used, but there are some problems in that reproducibility is not good, and the like due to inhibition of coloring of starch by iodine
20 by co-existing protein or due to short reaction time.

 As to the chromogenic method, the blue starch method is generally used, wherein there is used an insoluble substrate obtained by combining starch or amylose with a dyestuff and a soluble dyestuff produced
25 by an enzymatic reaction is measured. This method is

1 widely used recently, but there are many problems in
that the activity as substrate is weak, the reaction
system is ununiform due to insolubility, complicated
procedures are necessary and application to auto
5 analyzers is difficult, etc.

As to the saccharogenic method, the Somogyi
method is typical one, but there are problems in that
a higher value is obtained due to glucose in a sample,
the procedures are undesirably complicated, etc.

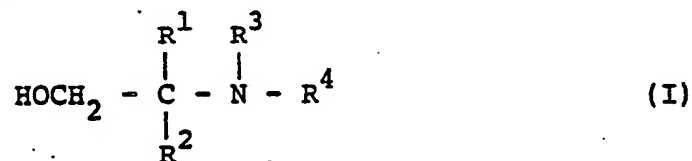
10 As mentioned above, individual methods for
measuring α -amylase activity have specific defects.
In addition, there are defects common to the above-
mentioned methods in that variations in measured
values are produced depending on the quality of starch
15 used as substrate, and the α -amylase reaction cannot
be measured as a true stoichiometrical reaction.

It is an object of this invention to provide
a process for measuring α -amylase activity precisely
by a rate assay or by using an auto analyzer. It is
20 another object of this invention to provide a process
for measuring α -amylase activity precisely by a fixed-
time assay using a special stopper and a reagent com-
position used therefor.

This invention provides a process for measuring
25 α -amylase activity comprising acting α -amylase on a
substrate, followed by acting a coupling enzyme on the
resulting decomposed substrate to produce glucose, and
determining quantitatively the glucose produced,

1 characterized by using as substrate a modified amylose
having one carboxymethyl group or a salt thereof per 6
to 35 glucose units in amylose.

This invention also provides a process for
5 measuring α -amylase activity using a substrate and a
coupling enzyme and determining quantitatively glucose
produced, characterized by using glucoamylase as
coupling enzyme, a modified substrate as substrate and
a reaction stopper of the formula:



wherein R^1 through R^4 are independently hydrogen or a
lower alkyl group having 1 to 4 carbon atoms, said
10 alkyl group being able to contain a hydroxyl group, or
a salt of said stopper, for stopping an enzymatic
reaction caused by glucoamylase.

In the attached drawings, Fig. 1 shows a
calibration curve for measuring α -amylase activity,
15 and Fig. 2 shows changes of absorbance before and after
addition of a stopper.

α -Amylase is an enzyme which hydrolyzes
 α -1,4-glycoside bonds. In starch or amylopectin having
 α -1,6-glycoside bonds, the α -1,6-glycoside bonds inhibit
20 the α -amylase reaction. On the other hand, amylose
is better than starch and amylopectin as substrate
since it is better in uniformity from the viewpoint of
chemical structure, which results in improving measuring

1 accuracy.

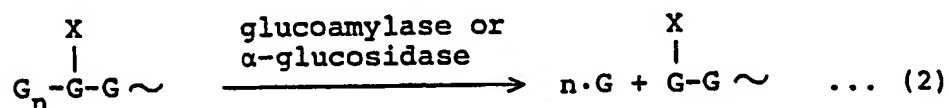
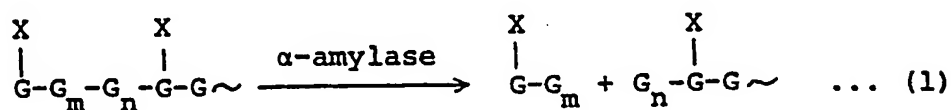
But when amylose is used as substrate in a process for measuring α -amylase activity by using glucoamylase or α -glucosidase as coupling enzyme and
5 determining quantitatively glucose produced, there arises a defect in that glucoamylase or α -glucosidase decomposes the substrate irrespective of the α -amylase reaction, since glucoamylase or α -glucosidase is an
10 exo type enzyme which hydrolyzes an α -1,4-glycoside bond from a non-reducing end of amylose irrespective of α -amylase. As a result, when amylose is used as substrate, there are many disadvantages in that a reagent solution for measurement cannot contain both the
15 substrate and glucoamylase or α -glucosidase at the same time, a sufficient amount of glucoamylase or α -glucosidase for measurement cannot be used, a reagent blank value increases remarkably, it is difficult to establish the measuring method with accuracy.

But the disadvantages mentioned above are
20 overcome by using a modified amylose having one carboxymethyl group or a salt thereof per 6 to 35 glucose units in amylose (hereinafter referred to as "modified amylose") as substrate. The salt of carboxymethyl group means one which can give a carboxymethyl ion
25 when dissolved. Examples of such salts of carboxymethyl group are salts of alkali metals such as Na, K, Li, etc., an ammonium salt, etc.

The modified amylose is excellent in affinity

- 1 to α -amylase and can be a good substrate for α -amylase
 but cannot be a substrate for glucoamylase or
 α -glucosidase. Further, there can be obtained various
 advantages in that solubility for water increases
 5 remarkably, the substrate can be used in sufficient
 concentration suitable for the α -amylase reaction,
 stability in an aqueous solution is improved, and the
 like.

- Since glucoamylase or α -glucosidase is an
 10 exo type enzyme, the modified amylose cannot be used
 as a substrate. In contrast, α -amylase is an endo
 type enzyme which hydrolyzes α -1,4-glycoside bonds in
 amylose at any portions, and thus the modified amylose
 can be used as substrate. The modified amylose is
 15 hydrolyzed by the enzymatic action of α -amylase to
 newly form non-reducing ends. On the newly formed non-
 reduced ends, α -glucosidase or glucoamylase acts to
 produce glucose. By determining the amount of glucose
 produced, there can be measured α -amylase activity.
 20 These reactions can be shown as follows:

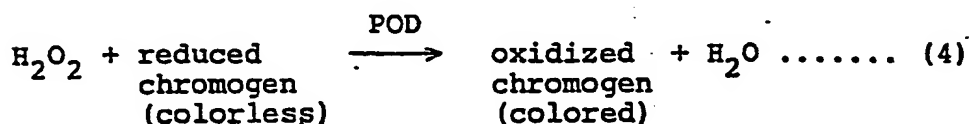
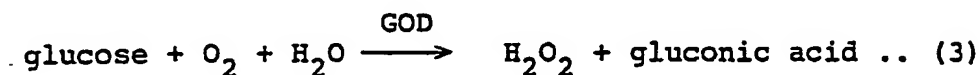


In the above sequence, G represents a glucose unit, X

1 is a carboxymethyl group or a salt thereof, and n and
m are independently integers of 2 or more.

There have been known various methods for
quantitatively determining glucose, which methods can
5 be used in this invention. Major methods for quantita-
tively determining glucose are shown below.

One method is as follows. When glucose is
acted by glucose oxidase, it is oxidized to produce
hydrogen peroxide. The produced hydrogen peroxide
10 oxidizes a chromogen quantitatively by means of
peroxidase present to give an oxidized chromogen. The
amount of glucose in the reaction solution can be
measured by colorimetric determination of the color
produced by the oxidized chromogen. These reactions
15 can be shown below.

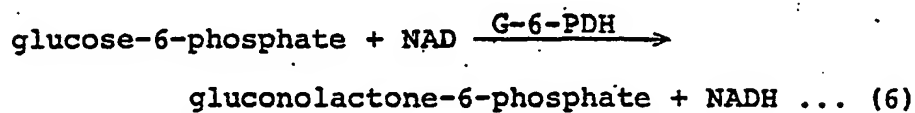
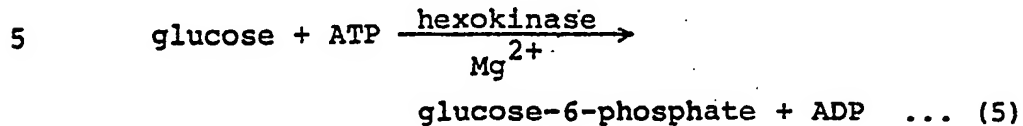


GOD: glucose oxidase

POD: peroxidase

Another method is as follows. Glucose is
changed to glucose-6-phosphate in the presence of ATP
20 by hexokinase. The produced glucose-6-phosphate is
changed to gluconolactone-6-phosphate in the presence
of NAD by glucose-6-phosphate dehydrogenase. On the

1 other hand, NAD is reduced to NADH. By measuring an increase in absorbance of NADH at near 340 nm, the amount of glucose in the reaction solution can be determined. These reactions can be shown below.



ATP: adenosine triphosphate

10 ADP: adenosine diphosphate

NAD: nicotinamide adenine dinucleotide

NADH: nicotinamide adenine dinucleotide, reduced form

G-6-PDH: glucose-6-phosphate dehydrogenase

15 In the process of this invention wherein a sample is treated with α -amylase and simultaneously or succesively with a coupling enzyme such as glucoamylase or α -glucosidase using the modified amylose as substrate and glucose produced is determined quantitatively,

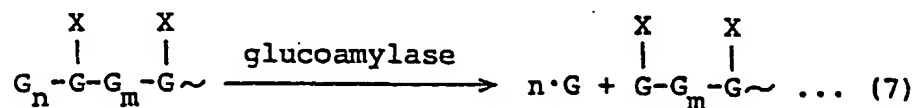
20 there is a possibility of influence of glucose present in a sample, particularly in a living sample such as serum, urine, etc. It is an effective means to remove or extinguish the glucose present in a sample before the α -amylase reaction in practicing the process of this

1 invention.

As to the removing or extinguishing method of glucose in the sample, there can be used a method of using glucose oxidase-catalase, a method of using
 5 hexokinase (Japanese Patent Appln Kokai (Laid-Open) No. 47495/82), a method of using glucose peroxidase, and the like, alone or in combination of these methods.

The modified amylose can be produced by reacting amylose having a molecular weight of about
 10 3,000 to 200,000, sodium hydroxide and monochloroacetic acid in an aqueous solution. It is preferable to use 5 to 30 moles of alkali such as NaOH and 0.5 to 5 moles of monochloroacetic acid per mole of glucose unit in amylose. The reaction is preferably carried out at
 15 about 30 to 70°C for 30 minutes to 3 hours with stirring. (See the process disclosed by S. Peat et al in "Nature" vol. 159, p. 810 (1947).)

Then, the product is neutralized and then treated with glucoamylase in an aqueous solution near
 20 neutral at 37°C for 10 to 30 hours to introduce a carboxymethyl group or a salt thereof into glucose units at non-reduced ends or near to said ends for giving the modified amylose. This reaction can be shown as follows.



1 In the above equation, G is a glucose unit, X is a
carboxymethyl group or a salt thereof, n is an integer
of 1 or more, and m is an integer of about 5 to
34.

5 In the above-mentioned reaction, the treatment with glucoamylase is not essential, but is effective to lower the level of blank value in measured values.

Then the reaction product is subjected to dialysis using water as outer liquid. By the dialysis,
10 by-products such as sodium chloride, sodium hydroxyacetate, etc., and glucose obtained by hydrolysis by glucoamylase are removed. After condensation, there can be obtained dried modified amylose. Degree of carboxymethylation of sodium salt of carboxymethylamylose
15 can be obtained by a pH titration.

The number of modifying group, i.e. the carboxymethyl group or a salt thereof which is introduced into amylose, is a very important factor from the viewpoint of precision and accuracy of α -amylase
20 activity measurement. Introduction of too many modifying groups into amylose will inhibit the action of α -amylase. When one or more modifying groups per 5 glucose units are introduced, the action of α -amylase decreases, which results in lowering in sensitivity,
25 precision and accuracy and making it impossible to use practically. On the other hand, introduction of too small modifying groups into amylose is not preferable, since contribution to improvement in solubility of

1 amylose by the modifying group is little and there
cannot be obtained a modified amylose having sufficient
solubility.

In order to maintain a necessary substrate
5 concentration, it is necessary to introduce one or more
modifying groups per 35 glucose units.

Therefore, as the modified amylose, there are
used those having one modifying group per 6 to 35
glucose units in amylose.

10 As a sample usable in the process of this
invention, there can be used any samples containing
 α -amylase. Examples of specimens are blood, serum,
urine, etc., as components in living body.

Glucoamylase or α -glucosidase derived from
15 animals and microorganisms can be used in this invention
but not limited thereto.

Measuring conditions for practicing this
invention are as follows. The reaction temperature of
the formulas (1) and (2) is not particularly limited
20 but preferably about 25 to 40°C. The reaction time can
be selected freely depending on purposes. The pH in
the reaction formulas (1) and (2) is not particularly
limited but preferably pH is about 6 to 8. In order
to maintain the pH at a preferable value, there can be
25 used buffers such as phosphate, Good's buffers.

In addition, there can be used an activator
of α -amylase such as sodium chloride, calcium chloride,
potassium chloride, etc.

1 As the measuring method of α -amylase activity,
there can preferably be used a rate assay in which a
reaction rate is measured under certain conditions.
It is also possible to use an fixed-time assay using a
5 reaction stopper but in this case it is preferable to
use an improved method mentioned below. The term
"fixed-time assay" means a method for measuring
enzymatic activity wherein there is determined a
changing amount of a substrate from an addition of a
10 stopper to a reaction solution after a prescribed
reaction time to a time at which the reaction is stopped
by said stopper.

By using the modified amylose as substrate
for measuring α -amylase activity, there can establish
15 measuring methods having the following advantages:

(i) Since the modified amylose is easily
soluble in water, there can be used any substrate
concentrations sufficient for the α -amylase reaction.
Thus, there can be obtained good calibration relationship
20 and wide measuring ranges.

(ii) Since the modified amylose cannot be a
substrate for glucoamylase and α -glucosidase and can
specifically be a substrate for α -amylase, no side
reaction takes place and reagent blank values are
25 extremely small.

(iii) Since a necessary and sufficient amount
of glucoamylase and α -glucosidase can be used, the
reactions after the α -amylase reaction are fast and

1 accurate rate assay becomes possible.

(iv) Thus, the glucose produced by the above mentioned reactions can be determined quantitatively by a conventional method with high sensitivity and good
5 precision.

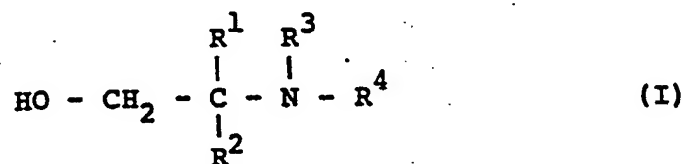
(v) Measuring procedures are simple.

(vi) Application to auto analyzers is easy.

(vii) Reagent solutions for measurement are stable.

10 In order to measure the α -amylase activity by the fixed-time assay (or one point assay) effectively, it is necessary to use a special reaction stopper which can stop effectively the reaction using the modified substrate.

15 Such a stopper is represented by the following formula (I) or a salt thereof.



In the above formula (I), R^1 through R^4 are independently hydrogen or a lower alkyl group having 1 to 4 carbon atoms, said alkyl group being able to contain a hydroxyl
20 group.

Since the stopper of the formula (I) or a salt thereof can effectively stop the activity of glucoamylase, the α -amylase activity can be measured

1 quantitatively with high accuracy and good reproducibility.

It has been reported that tris(hydroxymethyl)-aminomethane has an inhibition action against the
5 activity of glucoamylase. For example, according to an article recited in Agr. Biol. Chem., 41, 2149-2161 (1977), particularly tris(hydroxymethyl)aminomethane has an inhibitory action against the activity of glucomaylase. When 0.39 unit of glucoamylase derived
10 from Aspergillus awamori and 50 mM of tris(hydroxymethyl)-aminomethane are heated at pH 5.3 at 37°C for 5 minutes, followed by addition of soluble starch (0.2%) to measure residual activity from the amount of glucose produced, the residual activity is found to be 49.9%. According
15 to another article recited in Agr. Biol. Chem., 41, 2139-2148, (1977), glucoamylase derived from Mucor rouxianns is inhibited its activity by tris(hydroxymethyl)aminomethane in an amount of 5 mM at pH 5.3. The residual activity is found to be 26.7% or 28.8%.

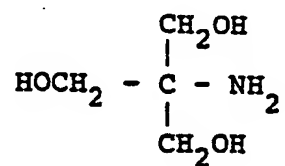
20 As mentioned above, the inhibitory action of tris(hydroxymethyl)aminomethane against the activity of glucoamylase is incomplete and considerable residual activity is admitted. On the other hand, in the fixed-time assay wherein an enzymatic reaction in the proceed-
25 ing reaction is stopped by a stopper, the stopper is required to stop the enzyme activity almost completely in a short time in order to give measurement results with high accuracy and good reproducibility. Therefore,

1 it is concluded that tris(hydroxymethyl)aminomethane
is not suitable for use as a stopper for glucoamylase
used in a proceeding reaction due to incomplete
inhibitory action.

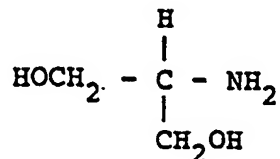
5 But the present inventors have noticed the
quality of substrate used and found that the inhibitory
action of a stopper against an enzyme is competitive
inhibition with a substrate used therein. Thus,
properly selecting a substrate, tris(hydroxymethyl)-
10 aminomethane can stop the activity of glucoamylase
effectively. The same effect can be shown in the
compounds of the formula (I) or salts thereof.

Examples of the reaction stoppers represented
by the formula (I) are as follows:

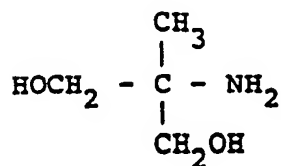
15 (i) Tris(hydroxymethyl)aminomethane (2-amino-2-
hydroxymethyl-1,3-propanediol)



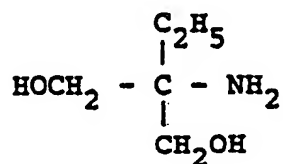
(ii) 2-Amino-1,3-propanediol



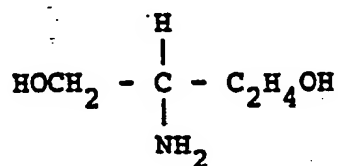
- 1 (iii) 2-Amino-2-methyl-1,3-propanediol



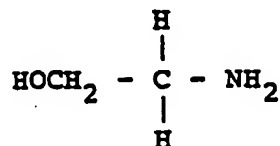
- (iv) 2-Amino-2-ethyl-1,3-propanediol



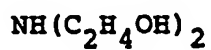
- (v) 2-Amino-1,4-butanediol



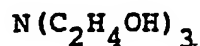
- (vi) Monoethanolamine



- 5 (vii) Diethanolamine



- 1 (viii) Triethanolamine



Examples of salts of the compounds of the formula (I) are salts of mineral acids such as hydro-
5 chloric acid, sulfuric acid, etc., or salts of organic acids such as maleic acid, oxalic acid, etc., of the compounds of the formula (I).

As the special substrate used together with stopper of the formula (I) or a salt thereof, there
10 can be used the modified amylose having one carboxymethyl group or a salt thereof per 6 to 35 glucose units in amylose as mentioned above. This substrate is particularly preferable from the viewpoint of solubility.

15 There can also be used as substrate modified amylose having 2-pyridylamino groups, 3-pyridylamino groups, anilino groups, 2-hydroxyanilino groups, methylanilino groups, carboxyphenylamino groups or the like modifying groups other than the carboxymethyl
20 groups, or polysaccharide having modifying groups at non-reducing ends in an amount suitable as substrate for α -amylase, e.g., sodium starch glycolate (carboxymethylated starch) (Clinica Chimica Acta, 76, 277-283 (1977)).

25 When polysaccharide having α -1,4-glycoside bonds such as starch or amylose is used as substrate, glucoamylase decomposes the substrate irrespective of

- 1 the α -amylase reaction in the method for measuring the
glucose produced by the enzymatic actions of α -amylase
and glucoamylase used as coupling enzyme, since glyco-
amylase is an exo type enzyme which hydrolyzes α -1,4-
5 glycoside bonds from non-reducing ends of amylose
irrespective of α -amylase. Thus, starch and amylose
conventionally used cannot be used as substrate.

Measuring conditions for carrying out the
fixed-time assay are as follows. Particularly effective
10 concentration of the stopper of the formula (I) or a
salt thereof at the time of reaction stoppage is 50
mM or more, preferably 100 mM or more up to its solu-
bility limit.

The reaction temperature of the α -amylase
15 reaction (1) and the coupling enzyme reaction (2) is
not limited particularly but preferably about 25 to
40°C. The reaction time can be selected freely depending
on purposes. The pH in the reaction formula (1) or
(2) is not particularly limited but preferably pH is
20 about 6 to 8. In order to maintain the pH at a preferable
value, there can be used buffers such as phosphate,
Good's buffers. Further, pH of the final liquid is
preferably 6.5 to 8.5, although slightly changes depend-
ing on the glucose determination system.

25 By the use of a special stopper of the formula
(I) or a salt thereof in combination with a special
modified substrate, the use of a special auto analyzer
is not necessary, a manual method can be used and

1 measuring methods practically useful are widened, and
a large number of specimens can be measured at one time
by a manual method. Further, since the reaction can be
stopped extremely rapidly, measuring error is small and
5 accurate values can be obtained with good reproducibility.

In addition, since a strong acid or strong alkali is
not necessary and a mild liquid with near neutral can
be used, it is easy to maintain stable conditions for
color formation.

10 This invention is illustrated by way of the
following Examples, in which all percents are by weight
unless otherwise specified.

Example 1

Preparation of Modified Amylose

15 Reagents

(1) Amylose

Amylose having an average molecular weight of
about 150,000.

(2) Sodium hydroxide solution

20 A 50% sodium hydroxide solution is prepared.

(3) Monochloroacetic acid solution

A 25% monochloroacetic acid solution is
prepared.

(4) Glucoamylase

25 Glucoamylase derived from Rhizopus.

1 Experimental method

To 10 g of amylose, 150 ml of water and 50 ml of the sodium hydroxide solution are added and mixed at 50°C for 1 hour with stirring. Then, 40 ml of the
5 25% monochloroacetic acid solution is added thereto dropwise and heated at 50°C for additional 1 hour. After cooling to room temperature, the resulting solution is neutralized with hydrochloric acid. Subsequently, 1000 units of glucoamylase is added thereto and
10 incubated at 37°C for about 15 hours.

In order to remove by-produced sodium hydroxide, sodium hydroxyacetate and glucose, the reaction mixture is placed in a dialysis tube and subjected to dialysis using deionized water as outer liquid for 50 hours.

15 After condensing the resulting solution, the desired modified amylose is obtained by freeze-drying. The yield is 90%.

The degree of carboxymethylation of amylose is obtained by adding 0.1 N hydrochloric acid to a
20 prescribed amount of the resulting modified amylose and conducting a pH titration using 0.1 N sodium hydroxide. As a result, it is confirmed that one sodium carboxymethyl group per about 22 glucose units is introduced into the amylose.

25 Example 2Reagent

(1) Reagent solution (1)

1 In purified water, 40 mmole of Good's buffer
solution (PIPES), 50,000 units of glucoamylase, 100,000
units of glucose oxidase, 200 units of mutarotase,
500,000 units of catalase, 0.7 mmole of 4-aminoantipyrine,
5 15 mmole of sodium chloride and 5 mmole of calcium
chloride are dissolved. The resulting solution is made
pH 6.9 with sodium hydroxide and the whole amount is
made 1 liter.

(2) Reagent solution (2)

10 In purified water, 40 mmole of Good's buffer
solution (PIPES), 15,000 units of peroxidase, 15 mmole
of sodium chloride, 5 mmole of calcium chloride, 3 g
of modified amylose obtained in Example 1, 15 mmole of
sodium azide and 10 mmole of phenol are dissolved.
15 The resulting solution is made pH 6.9 with sodium
hydroxide and the whole amount is made 1 liter.

Measuring method

To 2 ml of the reagent solution (1), 20 μ l
of serum specimen is added and incubated at 37°C for
20 5 minutes. To this, 1 ml of the reagent solution (2)
is added and reacted at 37°C. Change of absorbance
($\Delta E/\text{min}$) at 505 nm is measured for 3 minutes from after
2 minutes to 5 minutes.

On the other hand, a calibration curve is
25 prepared by using standard specimens having known
 α -amylase activity and the same procedures as mentioned
above. Using this calibration curve, α -amylase activity

1 of the test specimen can be obtained.

The relationship between the α -amylase activity (Somogyi unit/dl) of standard specimens and the change of absorbance at 505 nm ($\Delta E/\text{min}$) is shown in Fig. 1.

5 The α -amylase activity of standard specimen is 500 Somogyi units.

Example 3

Reagents

(1) Reagent solution (3)

10 The same as the reagent solution (1) used in Example 2.

(2) Reagent solution (4)

The same as the reagent solution (2) used in Example 2.

15 (3) Reagent solution (5)

The same as the reagent solution (2) used in Example 2 except for using 0.3 g of amylose having an average molecular weight of 16,000 in place of 3 g of the modified amylose having sodium carboxymethyl

20 group.

(4) Serum specimen

Serum containing α -amylase having activity of 300 Somogyi units.

Measuring method

25 To 2 ml of the reagent solution (3), 20 μl of serum specimen is added. On the other hand, to 2 ml

1 of the reagent solution (3), 20 μ l of purified water is
 added. These specimens are incubated at 37°C for 5
 minutes. To each specimen, 1 ml of the reagent solution
 (4) is added and reacted at 37°C. Change of absorbance
 5 ($\Delta E/\text{min}$) at 505 nm is measured for 3 minutes from after
 2 minutes to 5 minutes. These results are measured
 values of the serum specimen and reagent blank using
 the modified amylose as substrate and shown in Table 1.

On the other hand, using the reagent solution
 10 (5) in place of the reagent solution (4), measured
 values are obtained in the same manner as mentioned
 above and the results are shown in Table 1.

Table 1

Substrate	Change of absorbance during 3 minutes ($\Delta E/\text{min}$)	
	Blank	Specimen
Modified amylose	0.006	0.372
Amylose (non-modified)	0.030	0.114

Example 4

Reagent

15 (1) Reagent solution (6)

In purified water, 10 mmole of Good's buffer
 solution (PIPES), 20,000 units of glucoamylase,

1 100,000 units of glucose oxidase, 200 units of mutarotase,
500,000 units of catalase, 1 mmole of N,N-diethylxylidine,
15 mmole of sodium chloride, and 5 mmole of calcium
chloride are dissolved. The resulting solution is made
5 pH 6.9 with sodium hydroxide and the whole amount is
made 1 liter.

(2) Reagent solution (7)

In purified water, 20 mmole of Good's buffer
solution (PIPES), 5,000 units of peroxidase, 15 mmole
10 of sodium chloride, 6 g of carboxymethylamylose prepared
in Example 1, 15 mmole of sodium azide, and 0.5 mmole
of 4-aminoantipyrine are dissolved. The resulting
solution is made pH 6.9 with sodium hydroxide and the
whole amount is made 1 liter.

15 (3) Reaction stopper solution (1)

In purified water, 0.6 mole of tris(hydroxy-
methyl)aminomethane is dissolved and the pH is made 7.5
with HCl, while making the whole amount 1 liter.

Measuring method

20 To 1 ml of the reagent solution (6), 10 μ l
of serum specimen is added and incubated at 37°C for
5 minutes. To this, 1 ml of the reagent solution (7)
is added and reacted at 37°C for 10 minutes. To the
resulting solution, the reaction stopper solution (1)
25 is added in an amount of 2 ml to stop the reaction and
absorbance at 620 nm is measured.

On the other hand, a calibration curve is

1 prepared by using standard specimens having known
α-amylase activity and the same procedures as mentioned
above. Using this calibration curve, α-amylase activity
of the test specimen can be obtained.

5 Fig. 2 shows changes of absorbance before and
after the addition of the reaction stopper solution (X)
to a serum specimen containing α-amylase having an
activity of 80 Somogyi unit/dl. As shown in Fig. 2,
the absorbance becomes constant after the addition of
10 the reaction stopper solution.

Example 5

Reagent

(1) Reagent solution (8)

In purified water, 20 mmole of potassium
15 phosphate, 30,000 units of glucoamylase, 10,000 units
of hexokinase, 10,000 units of glucose-6-phosphate
dehydrogenase, 5 mmole of magnesium chloride, 2 mmole
of NAD (nicotinamide adenine dinucleotide), 2 mmole
of ATP (adenosine triphosphate), and 3 g of carboxymethyl-
20 amylose are dissolved. The resulting solution is made
pH 7.0 with KOH and the whole amount is made 1 liter.

(2) Reaction stopper solution (2)

In purified water, 0.4 mole of tris(hydroxyl-
methyl)aminomethane is dissolved and the pH is made
25 7.0 with HCl, while making the whole amount 1 liter.

1 Measuring method

To 2 ml of the reagent solution (8), 20 μ l of serum specimen is added and incubated at 37°C for 10 minutes. To this, 1 ml of the reaction stopper solution
5 (2) is added to stop the reaction and absorbance at 340 nm is measured.

As specimen blank, a solution is prepared in the same manner as described in the reagent solution (8) except for not using carboxymethylamylose. Using
10 said solution, absorbance at 340 nm is measured in the same manner as mentioned above.

On the other hand, a calibration curve is prepared by using standard specimens having known α -amylase activity and the same procedures as mentioned
15 above, while removing the specimen blank value therefrom. Using this calibration curve, α -amylase activity of the test specimen is obtained.

Example 6Reagent

20 (1) Reagent solution (9)

In purified water, 10 mmole of Good's buffer solution (PIPES), 20,000 units of glucoamylase, 100,000 units of glucose oxidase, 100 units of mutarotase, 500,000 units of catalase, 1 mmole of N,N-
25 diethylxylylidine, 15 mmole of sodium chloride and 5 mmole of calcium chloride are dissolved. The resulting solution is made pH 6.9 with NaOH and the whole amount

1 is made 1 liter.

(2) Reagent solution (10)

In purified water, 20 mmole of Good's buffer solution (PIPES), 5,000 units of peroxidase, 15 mmole of sodium chloride, 6 g of carboxymethylamylose prepared in Example 1, 15 mmole of sodium azide and 0.5 mmole of 4-aminoantipyrine are dissolved. The resulting solution is made pH 6.9 with NaOH and the whole amount is made 1 liter.

10 (3) Reaction stopper solution (3)

In purified water, 0.6 mole (36 g) of mono-ethanolamine is dissolved and the pH is made 7.5, while making the whole amount 1 liter.

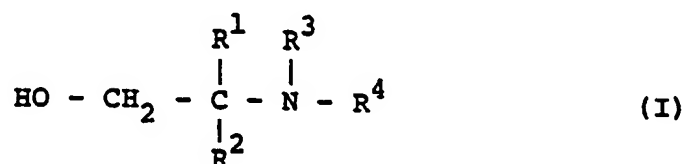
Measuring method

15 To 1 ml of the reagent solution (9), 10 μ l of serum specimen is added and incubated at 37°C for 5 minutes. To this, 1 ml of the reagent solution (10) is added and reacted at 37°C for 10 minutes. To the resulting solution, the reaction stopper solution (3) 20 is added in an amount of 2 ml to stop the reaction. Then, absorbance at 620 nm is measured.

On the other hand, a calibration curve is prepared by using standard specimens having known α -amylase activity and the same procedures as mentioned 25 above. Using this calibration curve, α -amylase activity of the test specimen can be obtained.

WHAT IS CLAIMED IS:

1. In a process for measuring α -amylase activity comprising acting α -amylase on a substrate, followed by acting a coupling enzyme on the resulting decomposed substrate to produce glucose, and determining quantitatively the glucose produced, the improvement which comprises using as the substrate a modified amylose having one carboxymethyl group or a salt thereof per 6 to 35 glucose units in amylose.
2. A process according to Claim 1, wherein the coupling enzyme is glucoamylase or α -glucosidase.
3. A process according to Claim 1, wherein the salt of carboxymethyl group is an alkali metal salt or ammonium salt of carboxymethyl group.
4. A process according to Claim 1, wherein the α -amylase activity is measured by a rate assay.
5. A process according to Claim 1, wherein the coupling enzyme is glucoamylase and the α -amylase activity is measured by a fixed-time assay using a reaction stopper of the formula:



wherein R^1 through R^4 are independently hydrogen or a lower alkyl group having 1 to 4 carbon atoms, said alkyl group being able to contain a hydroxyl group, or

a salt of said stopper for stopping the enzymatic reaction caused by glucoamylase.

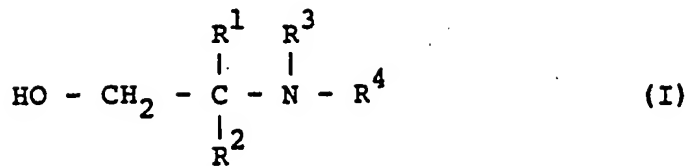
6. A process according to Claim 5, wherein the concentration of reaction stopper at the time of reaction stoppage is 50 mM or more.

7. A process according to Claim 5, wherein the reaction stopper of the formula (I) is 2-amino-2-hydroxymethyl-1,3-propanediol, 2-amino-1,3-propanediol, 2-amino-2-methyl-1,3-propanediol, 2-amino-2-ethyl-1,3-propanediol, 2-amino-1,4-butanediol, monoethanolamine, diethanolamine, or triethanolamine.

8. A process according to Claim 5, wherein the salt of the reaction stopper of the formula (I) is a mineral acid salt or organic acid salt of the reaction stopper of the formula (I).

9. A combination of reagents for measuring α -amylase activity by a rate assay, characterized by using a modified amylose having one carboxymethyl group or a salt thereof per 6 to 35 glucose units in amylose as substrate, and a coupling enzyme.

10. A combination of reagents for measuring α -amylase activity by a fixed-time assay, characterized by using a modified amylose having one carboxymethyl group or a salt thereof per 6 to 35 glucose units in amylose as substrate, glucoamylase as coupling enzyme and a reaction stopper of the formula:



wherein R¹ through R⁴ are independently hydrogen or a lower alkyl group having 1 to 4 carbon atoms, said alkyl group being able to contain a hydroxyl group, or a salt of said stopper.

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FIG. 1

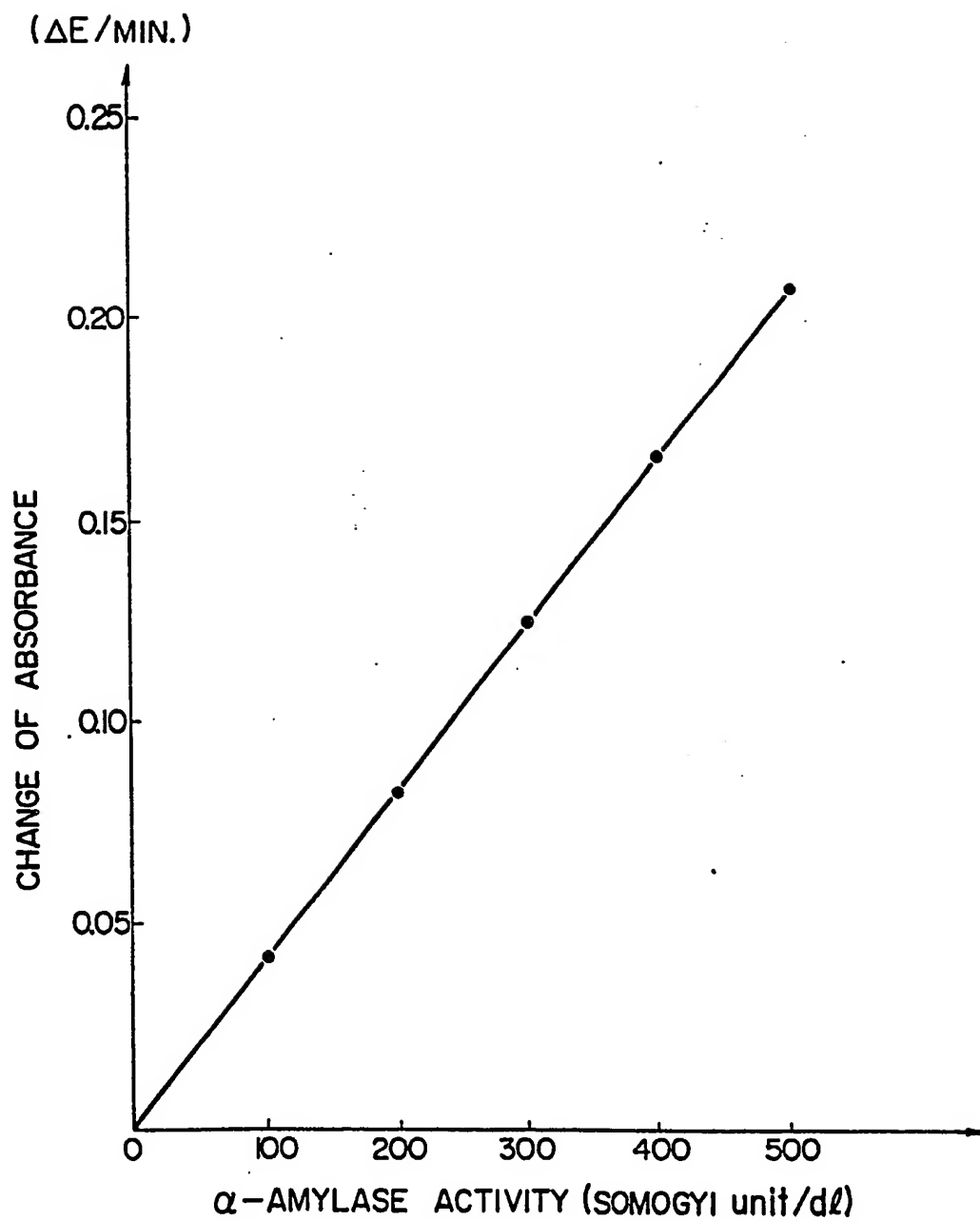
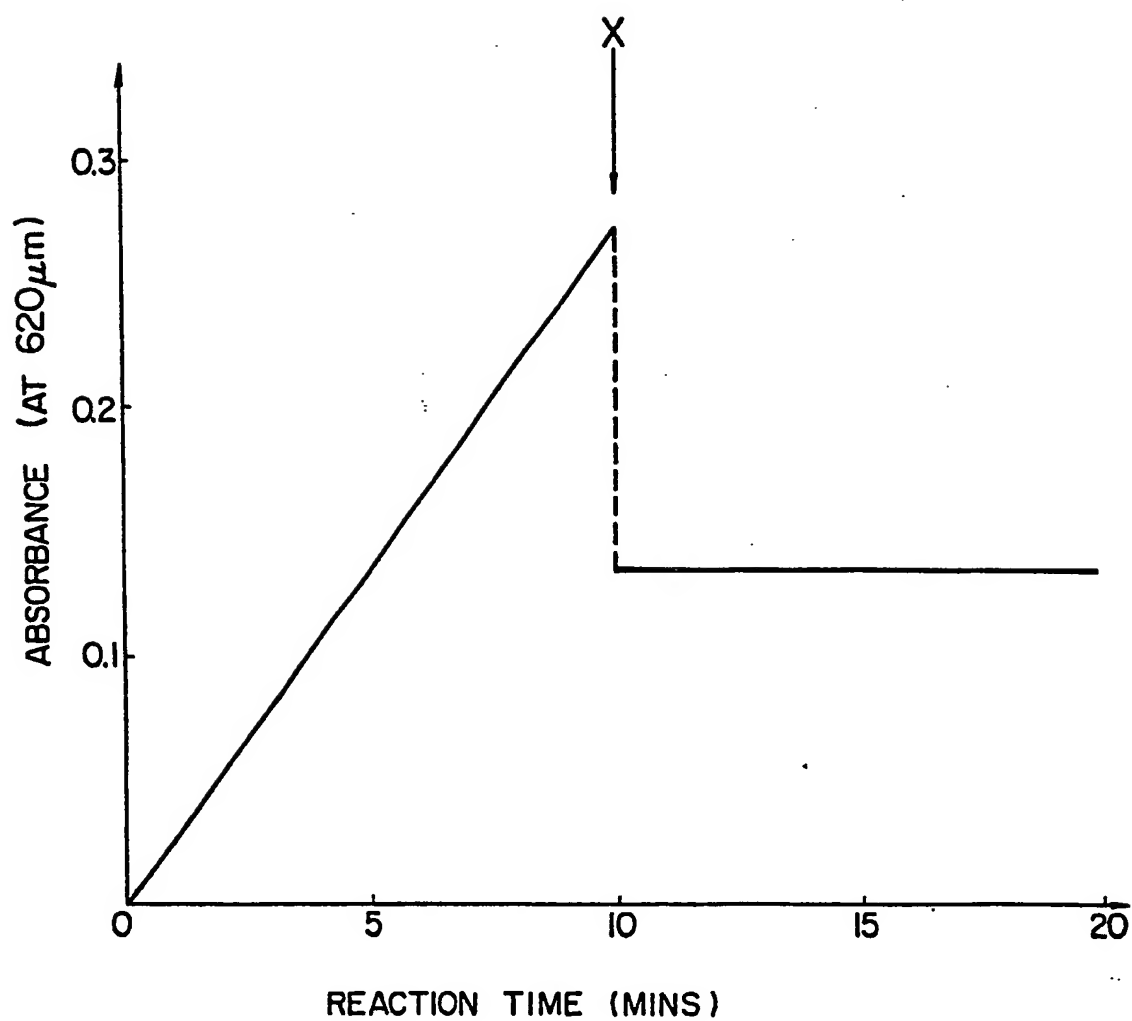


FIG. 2





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Place of search THE HAGUE		Date of completion of the search 06-12-1983	Examiner GRIFFITH G.
<p>CATEGORY OF CITED DOCUMENTS</p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document</p>			



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